ZOOLOGICAL SCIENCE 5: 347-351 (1988)

In vitro Dimerization of I-protein, an A-I Junctional Component of Skeletal Muscle Myofibrils[1]

MAKOTO TSUNEOKA[2], KOSCAK MARUYAMA and KAZUYO OHASHI[3]

Department of Biology, Faculty of Science, Chiba University, Chiba 260, Japan

ABSTRACT—Chicken myofibrillar I-protein, which was purified using ammonium sulfate precipitation and DEAE-cellulose column chromatography, was separated into two fractions by gel filtration, disc alkaline electrophoresis, or SDS polyacrylamide gel electrophoresis without SH reagents. These fractions consisted of 100,000 dalton and 50,000 dalton components. The amount of the high molecular weight component increased under the oxidizing conditions, while the amount of the low one increased when SH reagents were added. On the other hand, antiserum raised against 50,000 dalton component reacted with both of them, as revealed by immunoelectrophoresis. Therefore, it is concluded that I-protein dimerizes under oxidizing solutions. However, dimeric I-protein did not inhibit the ATPase activity of actomyosin *in vitro*, whereas monomeric I-protein did.

INTRODUCTION

I-protein is a myofibrillar protein which was isolated from chicken and rabbit striated muscles in 1977 [1-3]. The apparent molecular weight of I-protein, which was estimated from the migration rate of SDS polyacrylamide gel electrophoresis, is approximately 50,000 [1]. This protein is localized at the A-I junctional region of myofibrils in fresh myofibrils [4] and inhibits the ATPase activity of actomyosin *in vitro* [2].

In the process of chicken I-protein purification, we often found the coexistence of 100,000 dalton protein with I-protein in the I-protein fractions. We examined the form of I-protein in various solutions and found that the 100,000 dalton protein is a dimeric form of I-protein. It was observed that the inhibitory effect on actomyosin ATPase activity of these two forms of chicken I-protein were different.

Accepted October 20, 1987
Received August 11, 1987

[1] This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

[2] Present address: Department of Physiology, Kansai Medical University, Fumizono-cho, Moriguchi, Osaka 570, Japan.

[3] To whom reprint requests should be addressed.

MATERIALS AND METHODS

Preparation of I-protein and antiserum against I-protein

I-protein was prepared from chicken breast muscle according to the method described in a previous paper [1], using a DEAE-cellulose column. Antiserum against chicken I-protein was raised in a rabbit as mentioned before [4].

Electrophoresis

Disc alkaline electrophoreses of Tris-glycine buffer (pH 8.8) system were performed according to the method of Davis [5], using 10% acrylamide gel as separating gels (375 mM Tris-HCl, pH 8.8) and 4% acrylamide gel as stacking gels (125 mM Tris-HCl, pH 6.8). SDS polyacrylamide gel electrophoreses were carried out essentially according to Weber and Osborn [6], using 10% acrylamide gels with or without 2-mercaptoethanol (2-ME).

Determination of protein concentrations

Protein concentrations were determined by

means of biuret reaction or estimated from ultraviolet absorption at 280 nm and 260 nm in a Shimadzu spectrophotometer.

Immunoelectrophoresis

An immunodiffusion test of anti-I-protein antiserum against disc electrophoresed I-protein was carried out according to the method of Matsuda *et al.* [7]. A disc gel, on which I-protein was electrophoresed, was put on a slide glass and then 3 ml of 1% melted agarose containing 0.15 M NaCl and 20 mM sodium phosphate buffer, pH 7.2 was poured onto the slide glass. It was left for 30 min at room temperature. The agarose gel was grooved along the electrophoresed gel. Antiserum against I-protein was poured into the groove. This slide glass was placed in a moisture box overnight at room temperature. After dipped into PBS so as to remove soluble proteins, the slide glass was stained by 1% Amido Black in 7% acetic acid for 1 hr and subsequently destained by 4% acetic acid.

Oxidization and reduction of I-protein

Oxidized and reduced I-proteins were prepared according to the method described previously by Stewart [8]. I-protein was left for 2 hr at room temperature under an oxidizing condition: 1 M NaCl, 25 mM sodium borate buffer (pH 9.3), and 25 mM $CuCl_2$, or a reducing condition: 1 M NaCl, 25 mM sodium borate buffer (pH 9.3), 5 mM dithiothreitol (DTT). I-protein was also incubated in the same solution without $CuCl_2$ and DTT as control. Protein concentrations were finally adjusted to 0.2 mg/ml.

ATPase measurements

The ATPase activity of actomyosin was determined by measuring the amount of inorganic phosphate (Pi) liberated by the method of Taussky and Shorr [9]. The standard reaction mixture consisted of 43 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, and 10 mM Tris-HCl, pH 7.5. Incubation was carried out for 5–20 min at room temperature. The specific activity was given as μmoles of Pi split per mg of myosin per minute.

RESULTS

Monomeric and dimeric I-protein molecules coexisted in a solution

When a DEAE-cellulose column purified I-protein fraction [1] shown in Figure 1, a was applied onto the Sephadex G-200 column equi-

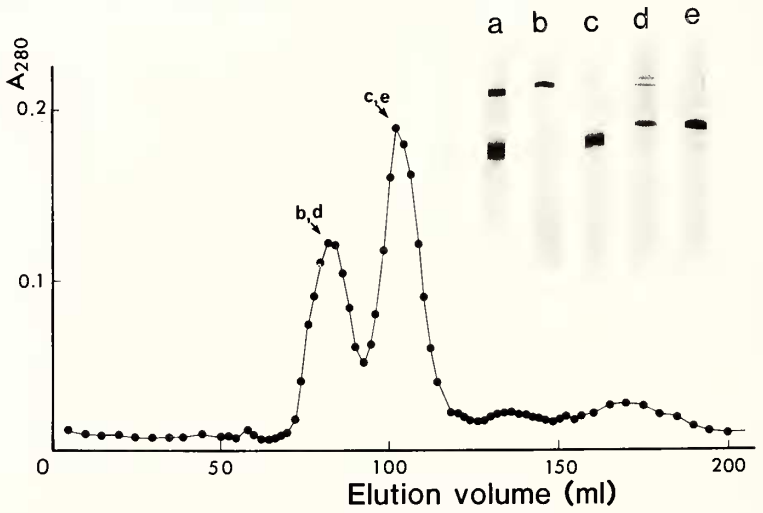

Fig. 1. Gel filtration chromatography of I-protein. Ion-exchange column purified I-protein was applied onto Sephadex G-200 column (1.8×90 cm). Fractionated proteins were electrophoresed by disc alkaline electrophoresis (a, b, c), or SDS-polyacrylamide gel electrophoresis with 2-ME (d, e). a; applied sample. b, d; protein eluted at 80 ml. c, e; protein eluted at 105 ml. Arrows indicate the electrophoresed fractions of b, d and c, e.

librated with 20 mM Tris-HCl, pH 7.5 and eluted with the same solution, the elution profile showed two peaks (Fig. 1). The first peak contained a protein of relatively low mobility by disc alkaline electrophoresis (Fig. 1, b) and a high mobility protein was eluted in the second peak (Fig. 1, c). These proteins in both peaks were electrophoresed with the same mobility on SDS polyacrylamide gels with 2-ME (Fig. 1, d, e). The ratio of the first peak to the second one calculated from the peak size was 5 : 8. Two protein bands on SDS polyacrylamide gels were electrophoresed at the positions of 100,000 dalton and 50,000 dalton proteins without any SH reagent (data are not shown). A small amount of two unidentified proteins, which molecular sizes were similar to that of 100,000 dalton protein, were contained in the first peak (Fig. 1, d).

Anti-I-protein antiserum reacted with both of the high and low mobility proteins

DEAE-cellulose column purified I-protein was electrophoresed on an alkaline disc gel. The gel was embedded in 1% agarose gel and reacted with anti-I-protein antiserum as described in Materials and Methods. A confluent immunoprecipitin line formed along the gel shows that the antiserum strongly reacted with both the high and the low mobility proteins of the same antigenicity (Fig. 2).

FIG. 2. Immunoelectrophoresis. Immunoelectrophoresis was carried out using 100 μg of I-protein and anti-I-protein antiserum. a, immunoelectrophoretic profile. The arrow head shows an immunoprecipitin line. b, disc alkaline electrophoresis of I-protein.

The ratio of the large molecular sized protein to the small one under a reducing condition differed from that under an oxidizing condition

Ion exchange column purified I-protein was incubated under oxidizing, reducing, or control conditions. The samples were electrophoresed in the system of Weber and Osborn without SH reagent. Each protein band stained with Coomassie Brilliant Blue R 250 was cut out and incubated overnight in 3 ml of 0.2% SDS and 0.2 M sodium phosphate buffer, pH 7.4, so as to extract the dye from acrylamide gel. The ratios of the large molecular sized protein were roughly estimated by measuring the absorbance of the dye solutions at 600 nm in a spectrophotometer. The sample left under an oxidizing condition contained more large molecular sized protein than the control sample. In the case of Figure 3, the ratio of the large molecular sized protein to the small one under an oxidizing condition was approximately 3 : 2 (Fig. 3, b), while the ratio of the control sample was 1 : 4 (Fig. 3, a). On the other hand, the sample under the reducing condition mainly contained the small molecular sized protein and did little large molecular sized one (Fig. 3, c). These two protein bands were electrophoresed at the position of 100,000 dalton and 50,000 dalton proteins. Therefore, it was concluded that these proteins were dimeric and monomeric I-proteins.

Dimeric I-protein possessed little inhibitory action on actomyosin ATPase activity

The inhibitory effects of I-protein in both

FIG. 3. SDS-polyacrylamide gel electrophoresis of oxidized and reduced I-protein. a, control I-protein. b, I-protein under an oxidizing condition. c, I-protein under a reducing condition.

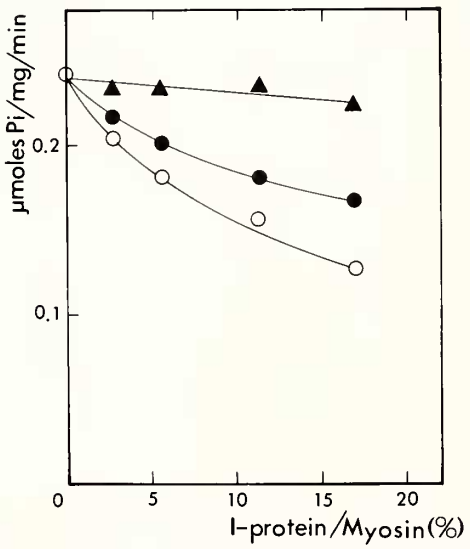

FIG. 4. The effect of dimeric or monomeric I-protein on the actomyosin ATPase. Ion-exchange column purified I-protein (○), dimeric I-protein (▲), or monomeric I-protein (●) was added to the actomyosin solution. The actomyosin ATPase was measured described in Materials and Methods.

dimeric and monomeric forms on actomyosin ATPase activity were examined (Fig. 4). Sephadex G-200 column fractionated dimeric and monomeric I-proteins (Fig. 1) were used. Reconstituted actomyosin was used. Final concentrations of myosin and actin were 0.8 mg/ml and 0.4 mg/ml, respectively. I-protein was added in various ratios to myosin. Dimeric I-protein hardly affected the actomyosin ATPase activity. Although the inhibitory action was lower than that of an original I-protein sample without gel-filtration, monomeric I-protein inhibited the actomyosin ATPase activity. Some denaturation of the protein during purification may account for the low inhibitory action of monomeric I-protein.

DISCUSSION

Ion exchange column purified I-protein usually containd both dimeric and monomeric molecules and the ratio of the two varied in each preparation. Dimeric I-protein usually increased with the lapse of time after preparation, even when stored at 0°C. In the present study, we showed that oxidization can account for the dimerization of I-protein. Dimerized I-protein was stable in a solution. Even an SDS solution containing 2-ME could not always dissolve the dimers into monomers in a short period incubation (data are not shown). However, the condition under which all monomeric I-protein molecules dimerize could not be specified.

Tropomyosin is a myofibrillar protein which also forms a dimer in a solution through disulfide bond [8]. Figure 3 shows that an oxidized I-protein solution contains more dimeric form and a reduced I-protein solution does more monomeric form. These results suggest that I-protein also forms a dimer through disulfide bond. It is assumed that I-protein may exist in a monomeric form under cellular circumstances. As is shown in Figure 4, dimeric I-protein lost the effect on the actomyosin ATPase activity while monomeric I-protein preserved. I-protein is localized at the A-I junctional region of myofibrils [4] and bind to myosin filaments *in vitro* [3]. These suggest that monomeric I-protein molecules bind to the both ends of thick filaments of myofibrils.

REFERENCES

1 Ohashi, K., Kimura, S., Deguchi, K. and Maruyama, K. (1977) I-protein, a new regulatory protein from vertebrate skeletal muscle I. Purification and characterization. J. Biochem., **81**: 233–236.

2 Ohashi, K., Masaki, T. and Maruyama, K. (1977) I-protein, a new regulatory protein from vertebrate skeletal muscle II. Localization. J. Biochem., **81**: 237–242.

3 Maruyama, K., Kunitomo, S., Kimura, S. and Ohashi, K. (1977) I-protein, a new regulatory protein from vertebrate skeletal muscle III. Function. J. Biochem., **81**: 243–247.

4 Ohashi, K., Tsuneoka, M. and Maruyama, K. (1985) I-protein is localized at the junctional region of A-bands and I-bands of chicken fresh myofibrils. J. Biochem., **81**: 1323–1328.

5 Davis, B. J. (1964) Disc electrophoresis – II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci., **121**: 404–427.

6 Weber, K. and Osborn, M. (1969) The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem., **244**: 4406–4412.

7 Matsuda, R., Obinata, T. and Shimada, Y. (1982) Types of troponin components during development of chicken skeletal muscle. Dev. Biol., **82**: 11–19.

8 Stewart, M. (1975) Tropomyosin: Evidence for no stagger between chains. FEBS Letters, **53**: 5–7.

9 Taussky, H. H. and Shorr, E. (1953) A microcolorimetric method for the determination of inorganic phosphorus. J. Biol. Chem., **203**: 675–681.